

ASPECTS OF MONITORING
PETROLEUM HYDROCARBON
CONCENTRATIONS BY HEPATIC
ARYL HYDROCARBON
HYDROXYLASE ACTIVITY IN
THE CUNNER, TAUTOGOLABRUS
ADSPERSUS (WALBAUM)
(PISCES: LABRIDAE)

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Aspects of Monitoring Petroleum Hydrocarbon Concentrations
by Hepatic Aryl Hydrocarbon Hydroxylase Activity in
the Cunner, Tautoglabrus adspersus (Walbaum) (Pisces:Labridae)

by

Douglas George Walton



A thesis submitted in partial fulfillment
of the requirements for the degree of

Master of Science

Department of Biology

Memorial University of Newfoundland

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Introduction

Oil discharge into the marine environment has been estimated at over 6 million metric tons per annum (National Academy of Sciences, 1975).

Hydrocarbon molecules comprise up to 98% of crude oil with the remainder being heterocyclic molecules containing oxygen, sulfur, and nitrogen (Speers and Whitehead, 1969; Nelson-Smith, 1973). A number of known carcinogens including benzo[a]pyrene and benzanthracene occur in oil (Medical Research Council, 1968; Pancirov and Brown, 1975) and Ermer (1970; *cf.* Matsushima and Sugimura, 1976) has shown that painting of 3-methylcholanthrene and benzo[a]pyrene on the skin of Gasterosteus aculeatus and Rhodeus amarus produced epitheliomas. In the aquatic environment there is an association between the presence of polycyclic aromatic hydrocarbons, and other pollutants, and carcinogenesis in fish (Dunn and Stich, 1976; Stich *et al.*, 1976; Sonstegard, 1977). It would therefore seem important to monitor the concentrations of aquatic hydrocarbons.

Conney *et al.* (1957) found that intraperitoneal injection of 3-methylcholanthrene and other polycyclic aromatic hydrocarbons resulted in an increase in the rate at which benzo[a]pyrene was hydroxylated by microsomes prepared from the livers of treated animals. The enzyme involved was subsequently called benzo[a]pyrene hydroxylase (E.C. 1.14.14.2) though more recent authors have termed it aryl hydrocarbon hydroxylase (AHH; E.C. 1.14.14.1), as its action on hydrocarbons became clarified. Benzo[a]pyrene hydroxylase has been most often studied in mammalian systems, especially in the rat (Gelboin, 1967). Hepatic basal activity levels in rats can be influenced by sex (Gurtoo and Parker, 1977) and nutrition (Kato and Gillette, 1965; Wiebel and Gelboin, 1975).

Though absent from all bivalves tested, AHH activity has been found in other molluscan classes and in annelids, arthropods, echinoderms, and chordates (Table 1) (Payne, 1977; Payne, 1978, personal communication). Payne and Penrose (1975), Payne (1976), Kurelec *et al.* (1977), and Stegeman (1978) have demonstrated the usefulness of AHH induction in fish as an indicator of marine petroleum hydrocarbon pollution. The cunner (Lutogolabrus adspersus Walbaum, 1792) used by Payne (1976) is a particularly good indicator species due to its behavioural characteristics. Green (1975) found the cunner to be a homing species which occupies relatively small home ranges. Through the winter months they remain in a torpid state in the inshore area (Green and Farwell, 1971) while in the summer months the cunner is found further inshore, frequently around wharves, and as it is readily attracted to food may be easily captured in large numbers. It ranges from Labrador to Chesapeake Bay although it is most abundant in the Gulf of St. Lawrence region (Leim and Scott, 1966).

Factors influencing drug-metabolizing capacity in fish have not been well studied. Buhler and Rasmusson (1968), Dewaide and Henderson (1970), and Dewaide (1971) could not discern, on the basis of sex, a difference in the rate of hepatic metabolism of the model substrates aniline and aminopyrine. More recently, Stegeman (1977), working with spawning rainbow trout (Salmo gairdneri) and the substrate aminopyrine, did observe such a difference. Nutritionally Buhler and Rasmusson (1968) noted no effect on hepatic mixed function oxidase activity when rainbow trout were starved or fed diets high in protein or carbohydrate.

As a further evaluation of the utility of hepatic aryl hydrocarbon hydroxylase activity in the cunner as a means of monitoring aquatic

Table 1. Presence of aryl hydrocarbon hydroxylase in marine phyla.

Phylum Chordata

Class Chondrichthyes

Raja sp. - Payne, 1977

Squalus acanthias - Malins, 1977

Class Osteichthyes

Tautoglabrus adspersus - Payne, 1977

Mallotus villosus - Payne, 1977

Pseudopleuronectes americanus - Pohl et al., 1974

Phylum Arthropoda

Class Crustacea

Cancer irroratus - Payne, 1977

Homarus americanus - Payne, 1977

Phylum Echinodermata

Class Asteroidea

Asterias sp. - Payne, 1977

Phylum Annelida

Class Polychaetae

Nereis sp. - Payne, 1977

Phylum Mollusca

Class Cephalopoda

Illex illecebrosus - Payne, 1977, personal communication

Class Gastropoda

Littorina littorea - Payne, 1977, personal communication

petroleum concentrations this study examined:

- (a) the effects of mode of oil exposure on induction of hepatic AHH;
- (b) seasonal, sex, and nutritional effects on activity in fish not exposed to inducing conditions (ie. "basal" levels);
- (c) the rates of induction (ie. increase in enzyme specific activity) and de-induction (ie. decrease in enzyme specific activity returning to the basal levels).

General Methods

I Collection and Handling of Experimental Fish

Cunners (Tautogolabrus adspersus), ranging in weight from 60-800 g, were collected from the mooring area at Portugal Cove (Fig. 1). Fish were transported in 70 L plastic containers to a 3500 L flow-through holding tank at the Marine Sciences Research Laboratory (MSRL), Logy Bay. From July through September, 1977, approximately fifteen collections of 50-60 fish each were made to maintain a stock of approximately 500 fish in the holding tank. The fish were fed commercially obtained capelin (Mallotus villosus) from a supply kept at -20 C at the MSRL. Though most cunners were held for several weeks prior to use, specific experiments utilized fish which had been maintained in the laboratory for one and two years as well as fish brought directly from the field.

II Experimental Procedure

Cunner dissection and enzyme assays were performed at the Physiological Ecology Laboratory at the Newfoundland Biological Station while oil exposure experiments were conducted at the MSRL. Venezuelan Tia Juana Medium Crude oil, provided by Golden Eagle of Canada Ltd., was used in all oil exposure experiments. The exposure tanks did not have temperature controls. However, the water temperature remained relatively stable (Fig. 2) over the duration of individual experiments.

III Measurement of Oil Concentrations in Water and Tissue

A. Oil Concentrations in Water

For all surface-slick exposures a time profile of oil concentration

Fig. 1. The cunner collection site at Portugal Cove.



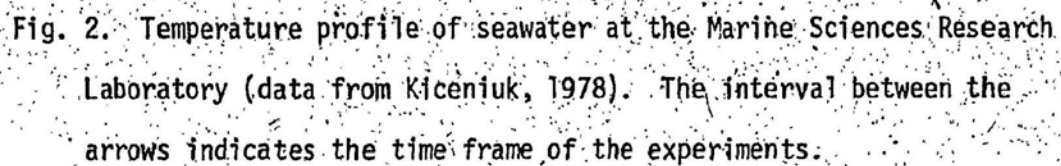
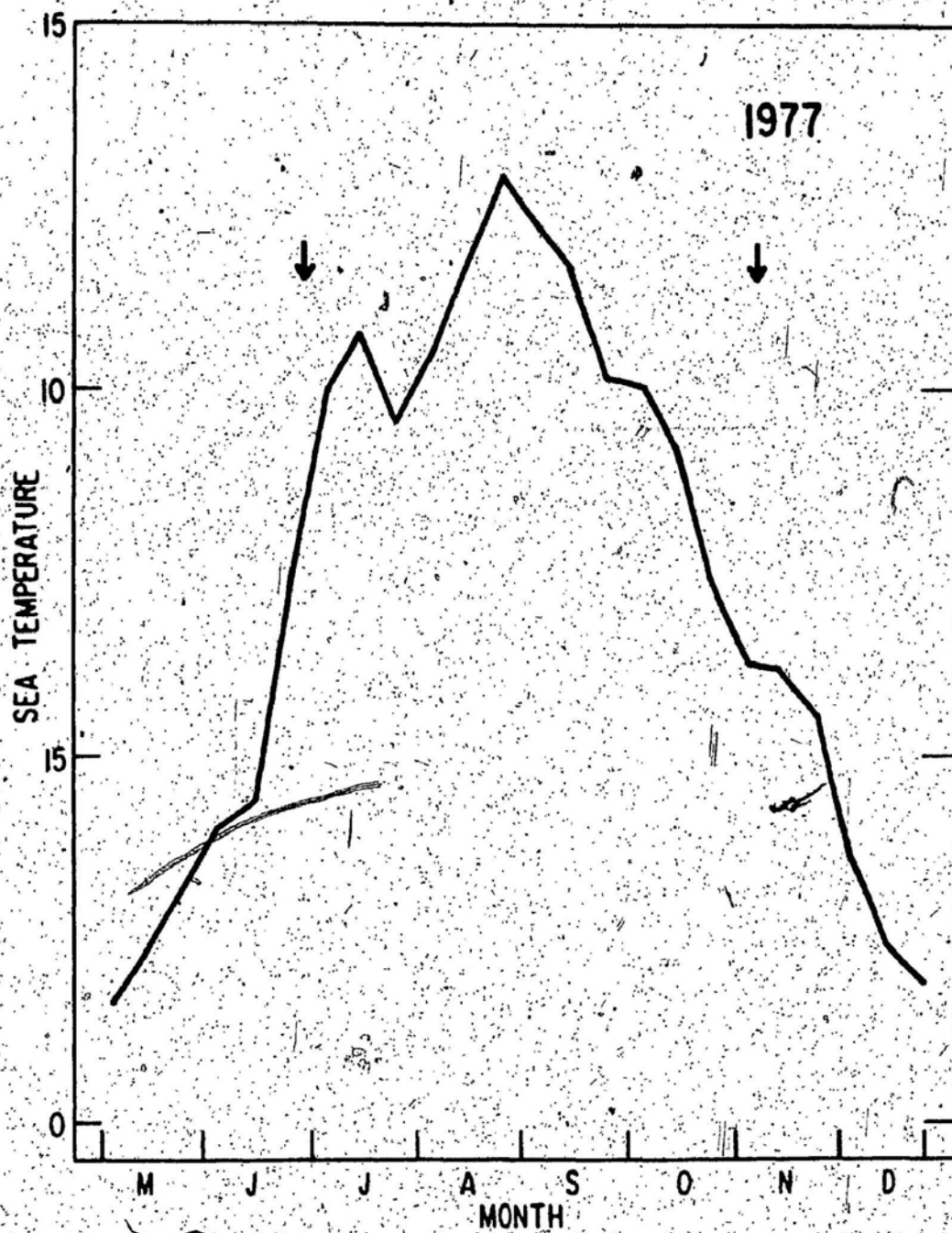


Fig. 2. Temperature profile of seawater at the Marine Sciences Research Laboratory (data from Kiceniuk, 1978). The interval between the arrows indicates the time frame of the experiments.



in the water was determined for the duration of the experiment. Oil concentrations were determined fluorometrically following a modification of the method of Keizer and Gordon (1973). 300 ml water samples were drawn into a solvent-washed B.O.D. bottle and extracted twice using 20 ml portions of methylene chloride. These samples were taken to dryness on a Büchi Rotavapor-R and the residue dissolved in 5.0 ml of isooctane. The fluorescence, using isooctane as a blank, was then measured using a Turner fluorimeter equipped with a Corning #7-60 excitation filter and #3 emission filter. A standard curve was constructed using fresh (unweathered) crude oil and the oil concentrations were expressed as parts per billion (ppb). All of the methylene chloride (Fisher, Spectrophotometric Grade) used for the extractions and rinsing all glassware was distilled from a flask containing a few grams of potassium permanganate to remove background fluorescence.

Oil concentrations were similarly determined for the force-fed water soluble fraction of crude oil (see p. 32). Oil concentrations were not determined for water in the force-feeding compartmentalized tank (see p. 32) as preliminary trials injecting 1.0 ml aliquots of oil below the surface showed that the oil rose quickly to the water surface and was swept away into the drainage trough.

B. Measuring Oil Concentrations in Mytilus Tissue

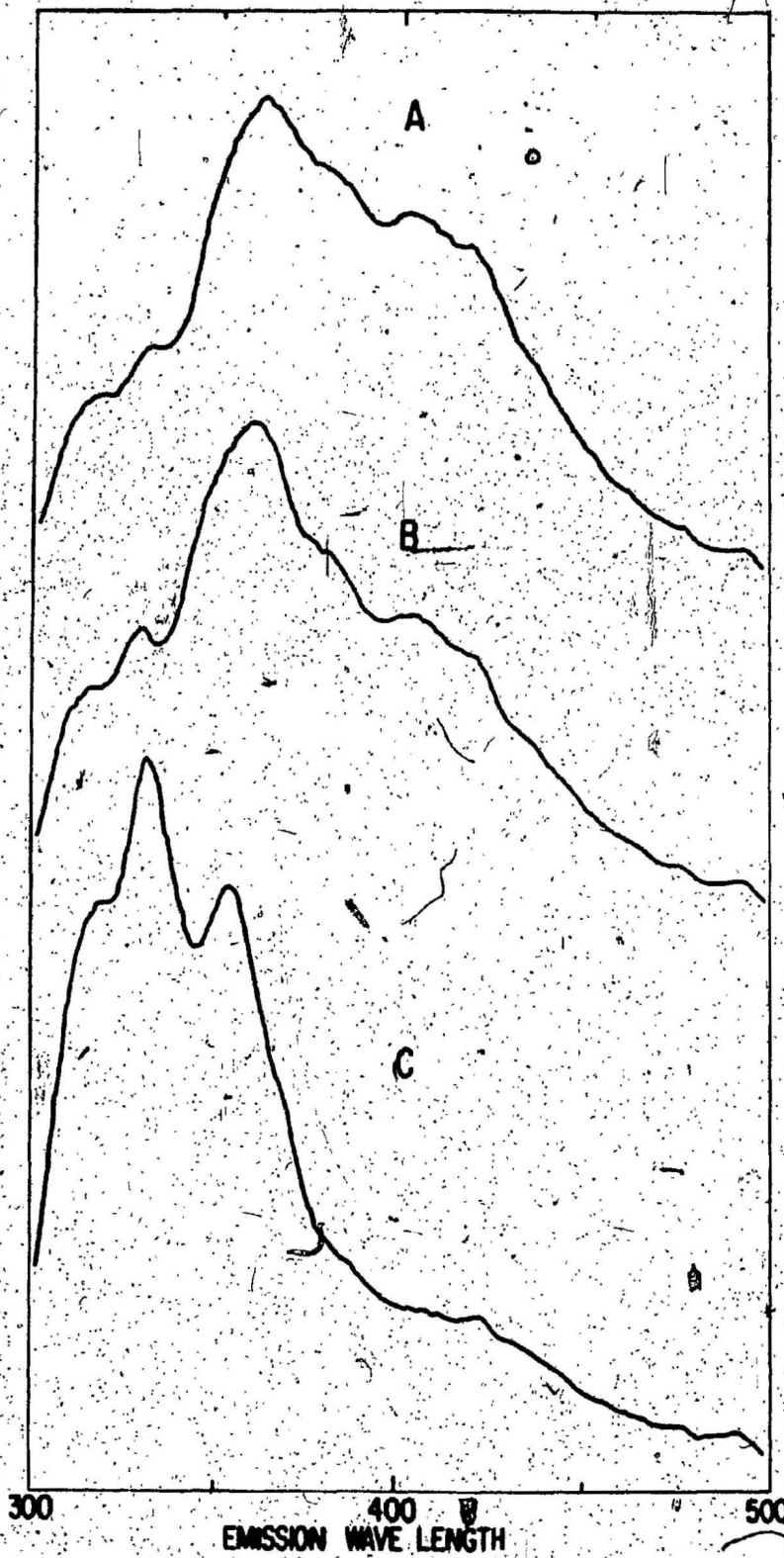
Oil concentrations in the homogenized Mytilus tissues were measured using ultraviolet and fluorescence methods (Vandermeulen *et al.*, 1977) by P. Ahern at the Marine Ecology Laboratory, Bedford Institute of Oceanography, Dartmouth, Nova Scotia. Samples packed in Dry Ice were shipped by air express to Dartmouth. Thawed samples were placed in extraction tubes and extracted into re-distilled methanol-benzene. The

resulting extract was saponified for two hours in methanolic KOH. After saponification each sample was put through a series of NaCl washes and pentane extractions to yield a single pentane-benzene phase. This was dried over anhydrous Na_2SO_4 and concentrated to less than 10 ml on a nitrogen evaporator. After making the samples up to 10 ml with isooctane, measurements were made by recording absorbance at 290 nm on a Unicam SP500 UV-visible spectrophotometer. From a calibration curve constructed from the Venezuelan crude oil the tissue concentrations of oil were obtained. Identity of the oil was established using synchronous fluorescence spectroscopy on the same extract (Fig. 3) (Vandermeulen *et al.*, 1977).

IV. Aryl Hydrocarbon Hydroxylase Activity Measurements

Fish were transported to the Physiological Ecology Laboratory and maintained for a few hours in 70-L plastic containers. For AHH measurements the liver was removed from fish freshly killed by concussion and a number of parameters such as sex, length, whole weight, liver weight, and state of gonadal maturity were recorded. A 0.5-4 g sample of liver was homogenized by hand using a Wheaton 15 ml pyrex tissue grinder with 10 ml sucrose buffer (0.05 M tris, 0.25 M sucrose, 1% KCl; pH 7.5). To limit enzyme degradation the homogenized tissue was kept on ice as much as possible. The homogenized tissue was centrifuged for fifteen minutes at 9000 x g using an International Equipment Company Model B-20A refrigerated centrifuge (0 C) and the supernatant was stored at -80 C in a Revco Ultra Low Freezer. Most homogenates were assayed within a week, however, on occasion it was impractical to assay homogenates immediately after preparation and a delay of up to 4 weeks was encountered. Assays,

Fig. 3. Synchronous excitation-emission fluorescence scans of isooctane extracts of Mytilus tissue. A: crude oil; B: extract of oil-contaminated Mytilus tissue; C: uncontaminated Mytilus tissue.



8 weeks apart, using both assay substrates (Table 2) demonstrate enzyme stability when stored under such conditions.

The AHH enzyme assay was essentially the fluorescence method of Nebert and Gelboin (1968) as modified by Payne and Penrose (1975). For use here the technique was further refined by determining the optimal range of protein concentration (Fig. 4) for the reaction. 1 ml sucrose buffer, 100 μ l of 1.6×10^{-3} mM NADPH, 20 μ l of 2 mM substrate in methanol, and 50 μ l homogenate were combined and incubated at 27 C for 10 minutes. The reaction was terminated with 2 ml of acetone. Both 2,5-diphenyl-oxazole (PPO) and benzo[a]pyrene (BP) were found to perform satisfactorily as substrates in the assay but, except for those experiments which compared the two substrates, PPO was preferentially used as it is not known to be carcinogenic. During the incubation, phenolic fluorescent metabolites of BP (3-hydroxybenzo[a]pyrene) and PPO (unknown metabolite) are formed. The BP metabolite is quite light sensitive (Payne, personal communication, 1977) while the PPO metabolite was found to not be light sensitive (Fig. 5). However, when the assay is carried out entirely in the light a slight decrease in activity is noted (Dawe, personal communication, 1978), suggesting that a reactive intermediate may be light sensitive. All assays were carried out under red safe-lights. Metabolites are isolated through extractions with 5 ml hexane, in which both metabolites and parent molecule are soluble, and a back-extraction with 5 ml of 1.0 N sodium hydroxide, in which only the phenolic metabolites are soluble as they can ionize. A good phase-separation was achieved by centrifuging for 4 minutes between extractions. Peak and spectral fluorescence were measured using a Perkin-Elmer Model 204 scanning fluorimeter. By exciting at 345 nm for PPO or 395 for BP and measuring the emission at

Table 2. Change in cunner hepatic aryl hydrocarbon hydroxylase activity assayed with both substrates at 0 and 8 weeks. The % changes are not significant at the .05 level of significance.

	Control		Induced	
	Mean Enzyme Activity (Std. Dev.)	Sample Size	Mean Enzyme Activity (Std. Dev.)	Sample Size
<u>PPO</u>				
0 Weeks	5.1 (1.66)	11	25.9 (9.29)	12
8 Weeks	5.0 (1.36)	5	24.6 (8.81)	10
% Change	(-) 0.8		(-) 5.2	
<u>BP</u>				
0 Weeks	3.8 (1.81)	11	71.8 (26.5)	12
8 Weeks	4.3 (1.51)	10	58.6 (20.2)	5
% Change	(+) 10.6		(-) 22.5	

Fig. 4 Effect of added protein on enzyme activity with both assay substrates. The assays were carried out using protein concentrations of 0.5 to 1.5 mg. (ie. within the linear portion of the curve).

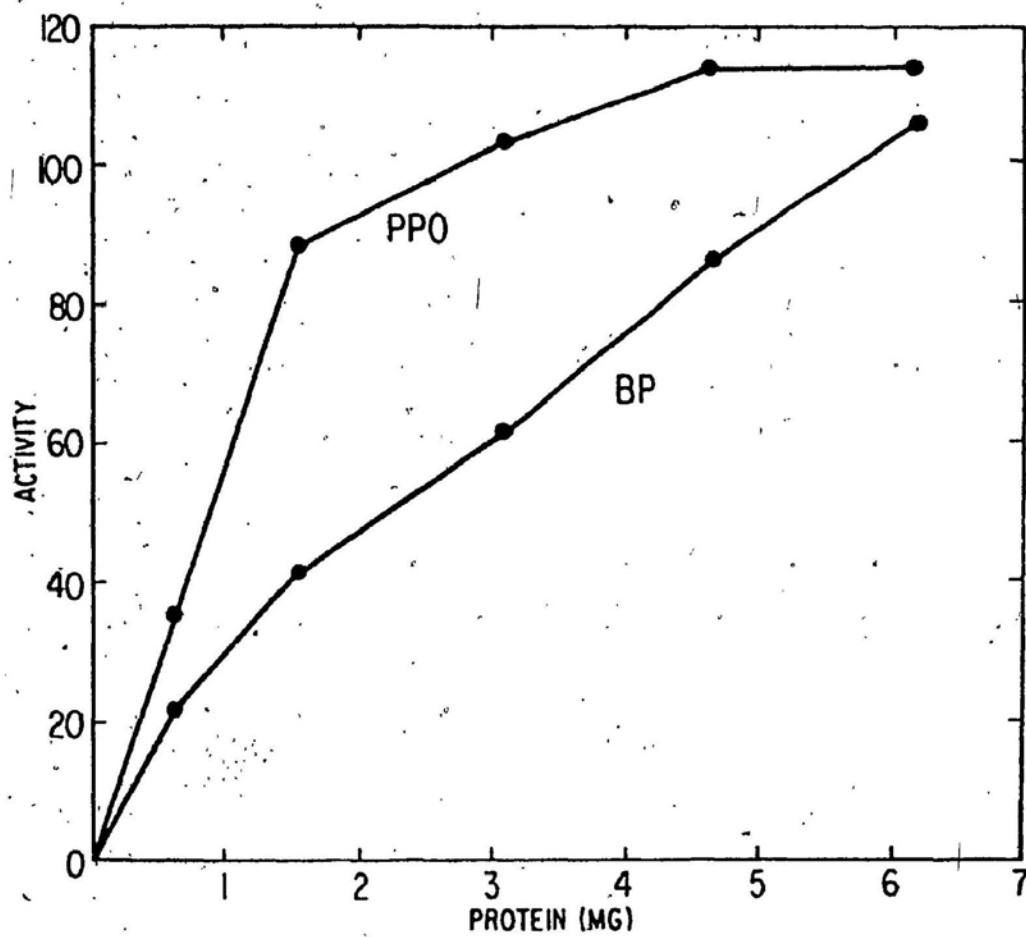
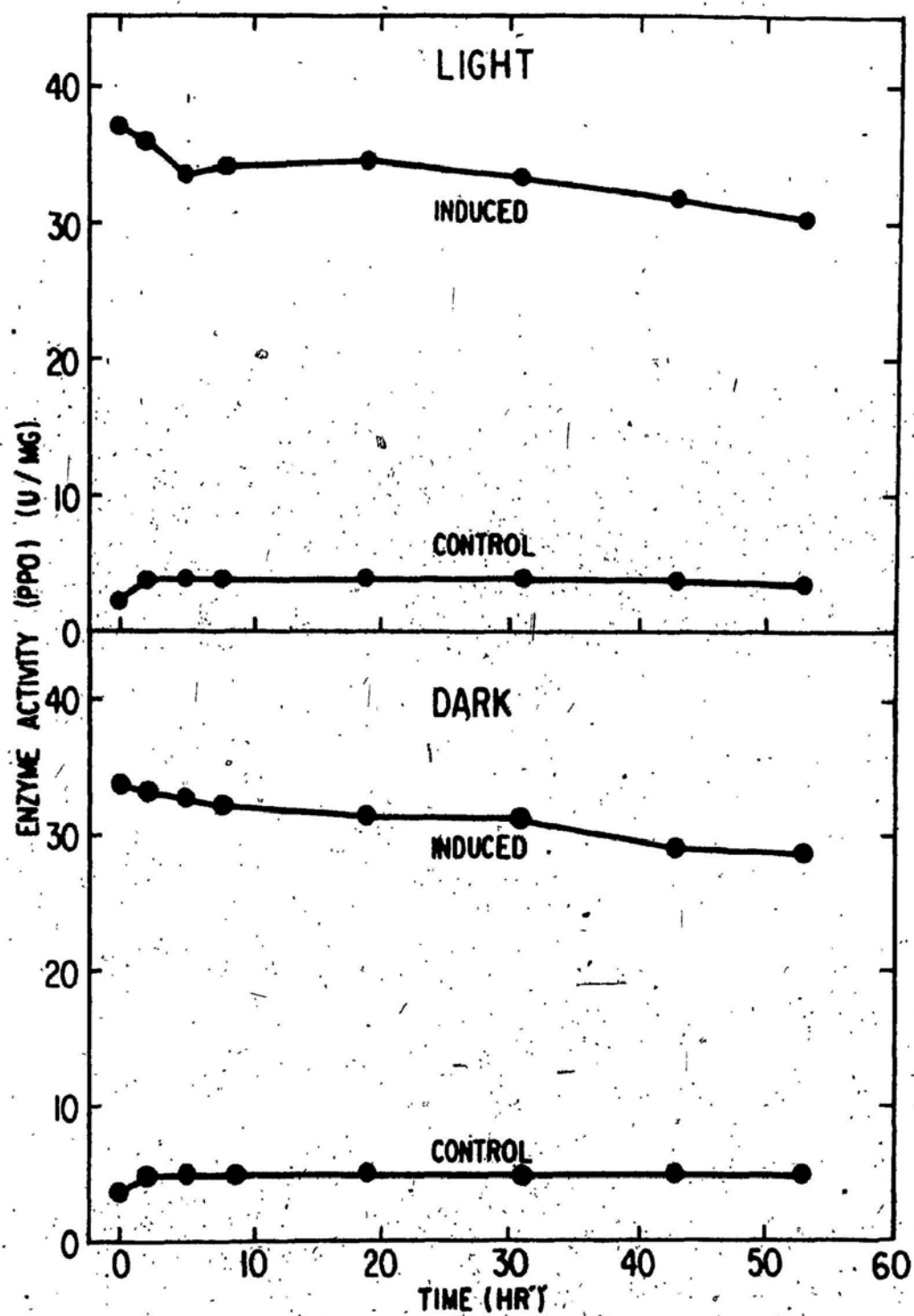


Fig. 5. Stability of the PPO metabolite under light and dark conditions.



510 nm or 520 nm respectively, unequivocal identification and quantification of the PPO and BP metabolites can be made. Individual homogenate assay replicability within and between assays was found to be fair (Table 3). Although a detailed study was not made, PPO assays seemed to provide better replication than BP. Protein concentrations of the homogenates were determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard. To allow for variation in tissue sample size and other uncontrollable factors, AHH activity was normalized to arbitrary units of alkali-extractable fluorescence per milligram protein (specific activity).

V Chemicals

The Tris and sucrose of the sucrose buffer used in the enzyme assays were Analar Grade (BDH, Poole, England), while the KCl was Fisher, Spectrophotometric Grade (Fairlawn, New Jersey). The NADPH used in the enzyme assay incubation was a chemically reduced tetrasodium salt, Type X (Sigma Chemical Co., St. Louis, Missouri). PPO was obtained from BDH while BP was from the Sigma Chemical Co. The hexane, octane, and acetone used were Fisher Spectrophotometric Grade and sodium hydroxide was a Baker Analyzed Reagent (Phillipsburg, New Jersey).

VI Statistical Analysis

Significant differences were ascertained using Student's two-tailed t-test. A probability level of 0.05 was arbitrarily chosen for judging significance. The probability levels used were 0.05, 0.02, and 0.01 and values were quoted such that $0.05 > P > 0.02$ was expressed as $P < 0.05$.

Table 3. Assayed aryl hydrocarbon hydroxylase activity, within and between trials, using the substrate PPO and the same fluorimeter sensitivity level (S8x1).

Assay	Trial	Fluorescence
Assay I	Trial 1	S8x1 - 47
"	"	S8x1 - 50
Assay II	Trial 2	S8x1 - 47.5
Assay III	Trial 3	S8x1 - 50.5
% range	-	6.9

Specific Experiments and Results

I Examination of Basal Activity Levels

Three factors known to influence AHH activity in mammalian systems are the animal's sex, ambient levels of polycyclic hydrocarbons, and nutritional status. These variables were investigated in the cunner to see how great an influence on basal activity they would have.

To see if a sex difference exists in this species hepatic AHH assays were performed on fish collected directly from the field, and on fish held in the Marine Sciences Research Laboratory for one and two years (Table 4,5). The July 8 sample of 15 fish, which had been held for 2 years in the lab, and July 18 sample of 26 fish from Portugal Cove both showed significantly ($P < 0.01$) less female basal activity than male. Dissection observations showed the gonads of these fish to be ripe. The August 1 collection of 20 fish held for one year in the lab as well as field collections of 29 and 20 fish from Portugal Cove made on August 19 and September 29, respectively, failed to show sex differences in AHH basal activity. Many of these fish upon dissection were noted to be spent and their gonads were showing signs of regression. The sex difference occurred during the cunner's spawning period which extended from July 11, 1977, to August 9, 1977, with peak spawning activity from July 18 to July 22 as judged by spawning behaviour observations at St. Phillips by B. Pottle (personal communication). Although many of the fish in the August 19 Portugal Cove sample were spent, about 40% were still in spawning condition which permitted enzyme activity comparisons on the basis of ripe versus spent males and females. These comparisons did not yield significant differences hence the sex difference in enzyme activity cannot

Table 4. Seasonal change in cunner hepatic aryl hydrocarbon hydroxylase activity, using the substrate PPO, of fish collected at Portugal Cove.

Portugal Cove	Mean Enzyme Activity (Std. Dev.)	Average Liver Weight g	Average Body Weight g	Liver Somatic Index % (Std. Dev.)	Sample Size
<u>July 18</u>					
Ripe Males	20.5 (5.38)	-	262	-	8
Ripe Females	5.9 (2.79)	-	243	-	18
Combined	12.4 (8.50)	-	268	-	26
<u>August 19</u>					
Ripe Males	25.0 (8.20)	5.8	302	1.88 (0.36)	8
Spent Males	24.5 (3.69)	4.4	244	1.97 (0.44)	6
Ripe Females	20.7 (2.11)	7.6	375	2.11 (0.29)	4
Spent Females	25.9 (7.16)	4.8	237	2.19 (0.47)	11
Combined	24.5 (6.59)	5.4	276	2.05 (0.41)	29
<u>September 27</u>					
Spent Males	18.2 (4.41)	5.1	177	2.94 (0.91)	10
Spent Females	23.1 (13.01)	3.7	136	2.51 (0.79)	10
Combined	20.7 (9.80)	4.4	156	2.72 (0.86)	20

Table 5. Cunner hepatic aryl hydrocarbon hydroxylase activities from other laboratory and field collections.

	Mean Enzyme Activity (Std. Dev.)	Average Liver Weight g	Average Body Weight g	Liver Somatic Index % (Std. Dev.)	Sample Size
<u>Fish 2 Yr. In</u>					
<u>Laboratory</u>					
<u>July 8</u>					
<u>BP</u> : Male	15.3 (6.12)	-	236	-	8
Female	5.4 (2.71)	-	310	-	7
Combined	10.7 (6.94)	-	271	-	15
<u>PPO</u> : Male	15.5 (3.60)	-	same	-	same
Female	8.7 (3.67)	-	as	-	as
Combined	12.3 (4.95)	-	above	-	above
<u>Fish 1 Yr. In</u>					
<u>Laboratory</u>					
<u>August 1</u>					
<u>PPO</u> : Male	8.5 (3.12)	5.9	272	2.22 (0.62)	10
Female	9.6 (3.23)	5.5	273	2.02 (0.32)	10
Combined	9.0 (3.14)	5.7	272	2.13 (0.51)	20
<u>St. Phillips</u>					
<u>August 16</u>					
<u>PPO</u> : Male	16.5 (5.54)	2.0	121	1.66 (0.33)	4
Female	18.3 (9.20)	2.5	128	1.89 (0.38)	8
Combined	17.7 (7.90)	2.4	125	1.83 (0.37)	12

be explained merely on the presence or absence of gonadal products. As a sex difference in enzyme activity was demonstrated when the fish were ripe enzyme activities from all other experiments were separated on a sex basis to see if such differences occurred when other variables were examined.

Due to less fishing and ferry activity it would seem probable that St. Phillips would have a lower background hydrocarbon level than Portugal Cove. Although comparison (Table 4,5) of collections on August 16, at St. Phillips, and August 16, at Portugal Cove, show a significant ($P < 0.05$) difference between "combined" enzyme activities there are not significant differences between any of the male/female comparisons. A clear difference in enzyme activity which may have been attributable to water quality was not discernable.

To investigate the effect of starvation on basal AHH activity two groups of cunner, one fed to satiation with capelin and the other starved, were maintained in 270 L flow-through tanks from August 26 to October 24. The results (Table 6) show there was approximately a 40% decline in basal activity of the starved fish with both BP and PP0. Starvation had an effect on liver weight as shown in Fig. 6 where liver weight is plotted against body weight for starved and fed fish. There was also a significant ($P < 0.01$) difference between the liver somatic indices of the starved and fed fish; however, there was no significant difference between males and females within the fed or starved groups with regard to liver somatic index.

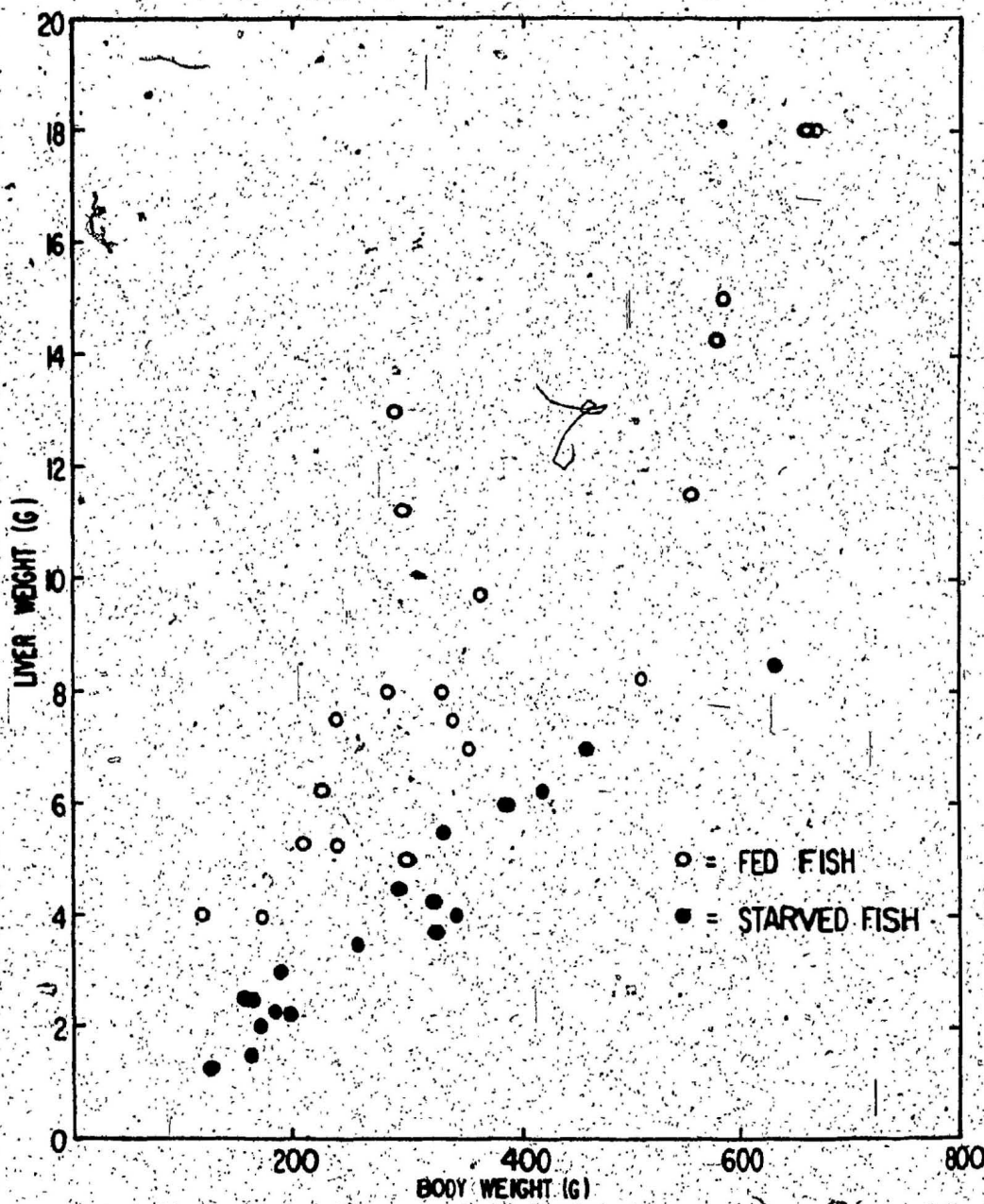
II. Surface Slick Exposures

Cunners were exposed to oil applied as a surface slick to establish

Table 6. Hepatic aryl hydrocarbon hydroxylase activity in cunners
starved and fed to satiation for 8 weeks.

	Mean Enzyme Activity (Std. Dev.)	Average Liver Weight g	Average Body Weight g	Liver Somatic Index % (Std. Dev.)	Sample Size
<u>Starved Fish</u>					
<u>PPO</u> : Male	5.0 (1.42)	3.3	239	1.33 (0.24)	5
Female	6.6 (1.77)	4.2	305	1.33 (0.21)	13
Combined	6.1 (2.53)	3.9	287	1.33 (0.21)	18
<u>BP</u> : Male	3.3 (1.24)	same	same	same	same
Female	5.2 (2.63)	as	as	as	as
Combined	4.7 (2.45)	above	above	above	above
<u>Fed Fish</u>					
<u>PPO</u> : Male	10.2 (1.73)	11.3	406	2.83 (0.67)	11
Female	10.2 (2.92)	6.9	317	2.37 (0.63)	9
Combined	10.3 (2.30)	9.3	366	2.62 (0.68)	20
<u>BP</u> : Male	8.5 (2.52)	same	same	same	same
Female	10.3 (3.12)	as	as	as	as
Combined	9.3 (2.88)	above	above	above	above

Fig. 6. Liver weight - body weight relationship for starved and fed cunners. Fed fish: slope = 0.023, intercept = 0.97, correlation coefficient = 0.87. Starved fish: slope = 0.015, intercept = -0.37, correlation coefficient = 0.97.



the quantity of oil needed to cause induction, to see if male and female fish induce at the same rate, and to determine the rate of induction and de-induction.

To estimate the amount of oil necessary to cause induction varying quantities of oil were added to five 20 L flow-through tanks each containing 7-9 fish. The flow rate was set at 2 L/min. and checked every 12 hours. Oil concentrations in the water were determined for the duration of the experiment, July 21 to July 31 (Fig. 7). Enzyme activity does increase with increased oil dosage (Table 7) but unfortunately these activities are difficult to compare as the experiment took place at a time when there was likely a sex difference present and there are not enough of either sex to provide a complete detailed comparison. However, examination of the "combined" data shows significant induction with 1 ml of oil. Comparing female activities, significant levels of induction only occur with 25 ml of oil.

The experiments examining cunner induction to oil and the rate of induction/de-induction were conducted using two 270 L fiberglass flow-through tanks (approximately 1m x 1m x 0.3m; Fig. 8), with polyethylene liners and covers and siphon drainage to prevent loss of floating oil. Each tank contained cement construction blocks to provide cover for the fish. The 2 L/min. flow-rate through the tanks and air supply were checked every 12 hours. The 200 ml of oil was applied with a Becton-Dickinson Plastipak syringe.

To determine if the rate of post-spawning induction is influenced by the individual's sex approximately 30 fish were placed in each tank (control and experimental) and the surface slick was applied. Oil concentrations in the water were determined during the exposure period,

Fig. 7. Variation in oil concentration during the 10-day surface slick exposure to differing quantities of oil. A = 0.2 ml; B = 1 ml; C = 5 ml; D = 25 ml; E = Control.

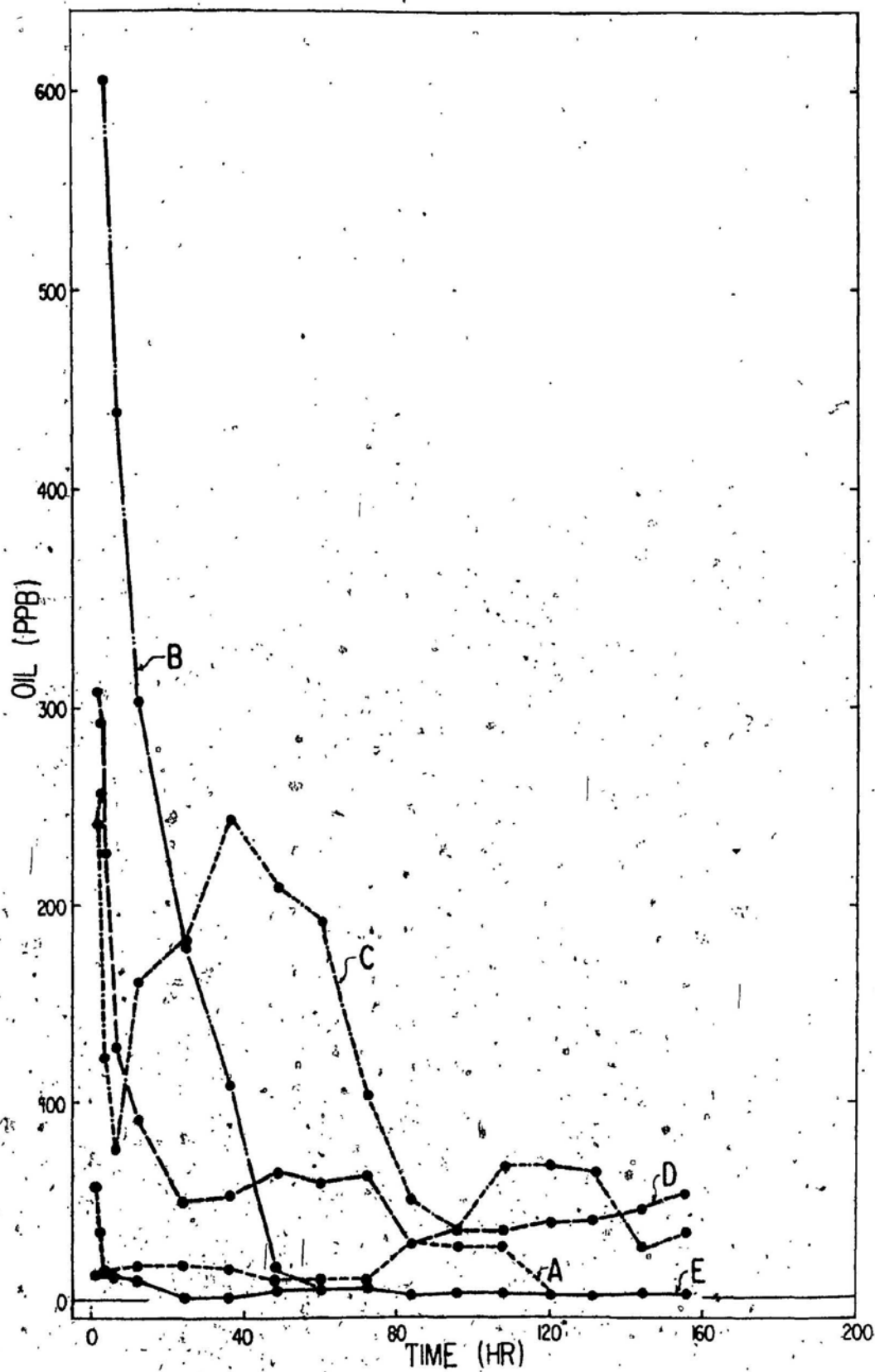


Table 7. Change in cunner hepatic aryl hydrocarbon hydroxylase activity, using the substrate PPO, following 10 days of exposure to varying quantities of oil.

		Mean Enzyme Activity (Std. Dev.)	Average Body Weight g	Sample Size
<u>Control:</u>	Male	11.3 (-)	151	1
	Female	8.2 (3.51)	222	7
	Combined	8.8 (3.65)	221	8
<u>0.2 ml Oil:</u>	Male	16.7 (0.21)	233	2
	Female	10.8 (1.15)	220	5
	Combined	12.5 (2.99)	223	7
<u>1.0 ml Oil:</u>	Male	16.5 (2.48)	193	5
	Female	17.9 (10.23)	240	3
	Combined	17.0 (5.83)	211	8
<u>5.0 ml Oil:</u>	Male	19.2 (9.74)	157	7
	Female	12.2 (3.04)	190	2
	Combined	17.5 (8.93)	163	9
<u>25 ml Oil:</u>	Male	28.5 (-)	167	1
	Female	16.5 (5.48)	157	6
	Combined	19.4 (9.06)	159	7

Fig. 8. The 270 L flow-through tanks used for the surface slick exposures.



August 14 to August 22 (Fig.9). The resultant enzyme activities (Table 8) showed significant differences ($P < 0.01$) between control and experimental fish but no sex differences within control or experimental fish. Male and female fish therefore appear to be induced at the same rate.

For the induction/de-induction experiment the flow-through tanks each contained 70-80 fish. A fourteen-day treatment period (oil present from September 3 to September 17) was followed by a twenty-day de-inductive (no oil from September 17 to October 7) period. The oil concentration profile (Fig. 10) indicates that at day fourteen oil-removal operations were successful in cleaning the exposure tank. The oil concentrations for the control tank ranged from 0 to 32 ppb and were omitted from this figure for clarity. Five control and five experimental fish were removed for enzyme analysis at each sample time during the inductive period. As there were fewer remaining control fish, four and five fish respectively were sampled for the de-inductive period. Figure II compares, through time, oil concentration with the corresponding enzyme activity. As surface slick exposure has already been shown to enhance enzyme activity a one-tail t-test was used to determine significant differences between pairs of control and experimental values. Examination of these significant differences showed that induction occurred within twenty-four hours and that the variation in the activity level correlated very well with oil concentrations in the water, assuming approximately a twenty-four time lag.

III Force-feeding of Oil and Oil-contaminated Mytilus Tissue

Uptake of hydrocarbons from a surface slick exposure need not be the only mode of exposure resulting in induced AHH activity. AHH induction

Fig. 9. Variation in oil concentration during the 8-day surface slick exposure. Solid line: Control; Dash-dot line: Experimental; Dashed line: 3-period moving average of the Experimental.

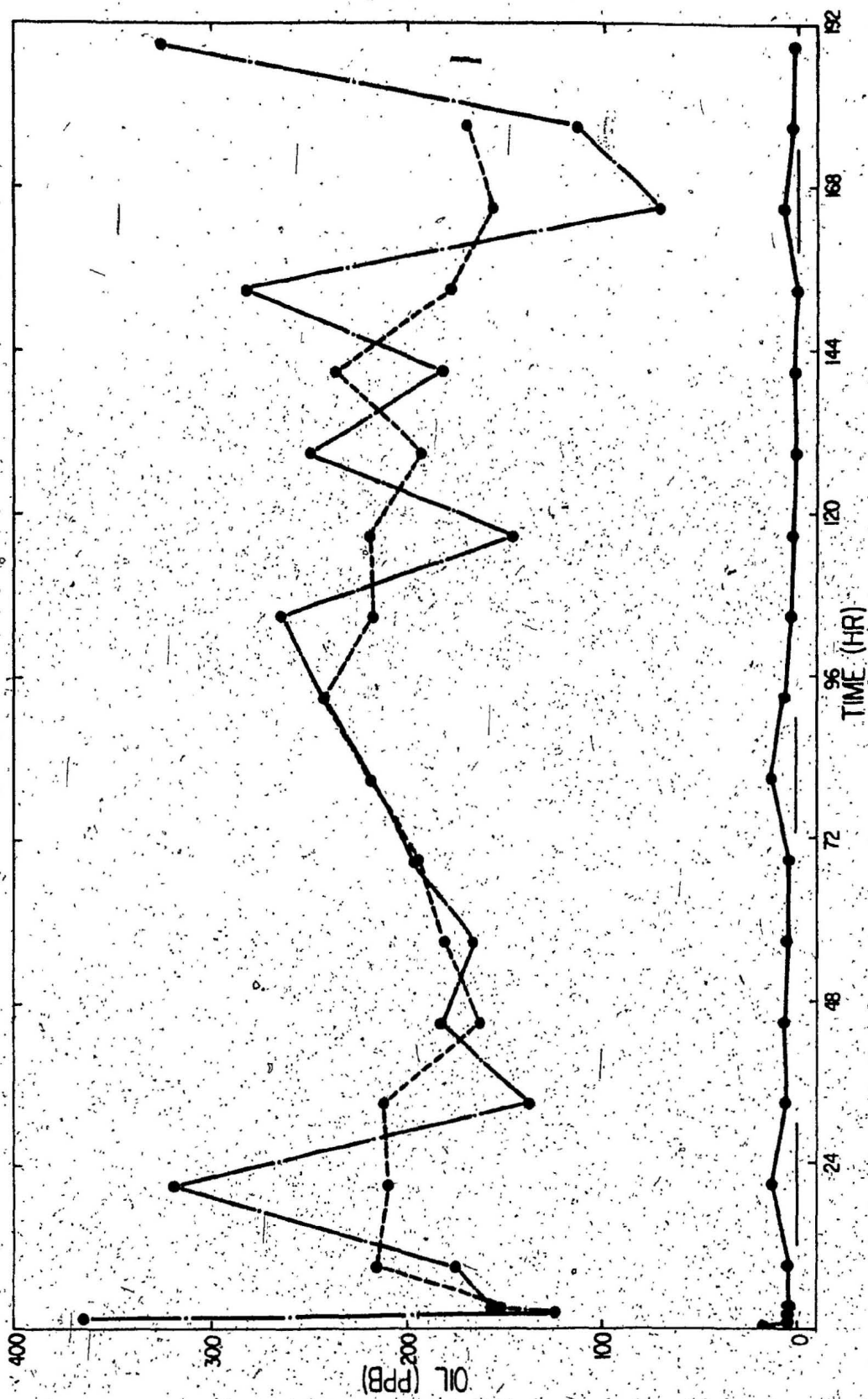


Table 8. Change in cunner hepatic aryl hydrocarbon hydroxylase activity, using the substrate PPO, following an eight-day surface slick exposure to oil.

	Mean Enzyme Activity (Std. Dev.)	Average Liver Weight g	Average Body Weight g	Liver Somatic Index % (Std. Dev.)	Sample Size
<u>Control:</u>					
Male	15.5 (3.99)	4.8	300	1.63 (0.28)	15
Female	14.5 (5.50)	4.9	288	1.62 (0.33)	15
Combined	15.0 (4.74)	4.8	294	1.63 (0.30)	30
<u>Experimental:</u>					
Male	22.5 (7.04)	3.0	209	1.40 (0.41)	18
Female	21.9 (6.03)	5.4	328	1.58 (0.37)	18
Combined	22.2 (6.47)	4.2	268	1.49 (0.40)	36

Fig 10. Oil concentrations in the water (solid line) from a surface slick, with a 3-period running average (broken line) to better indicate the trend over time.

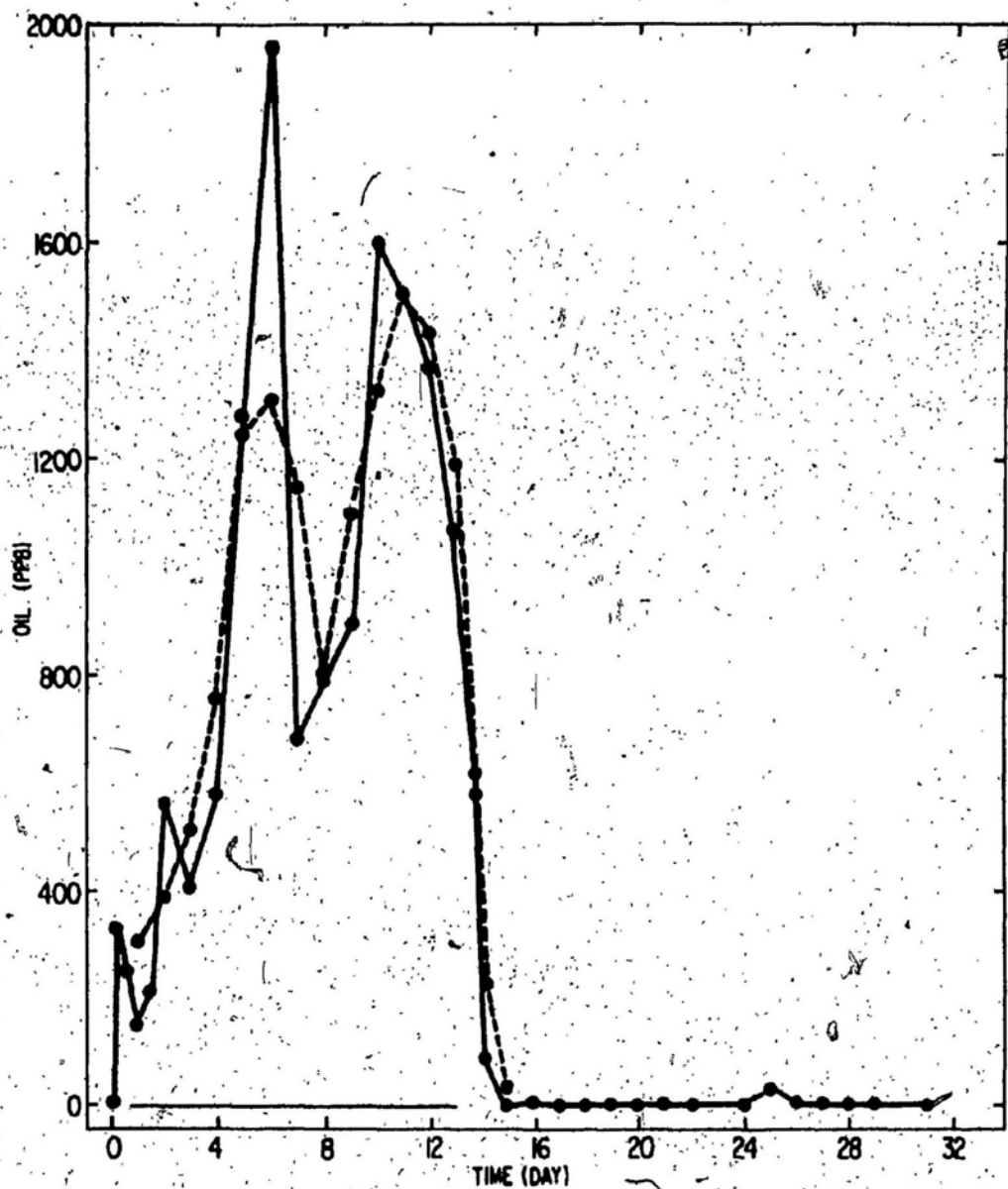
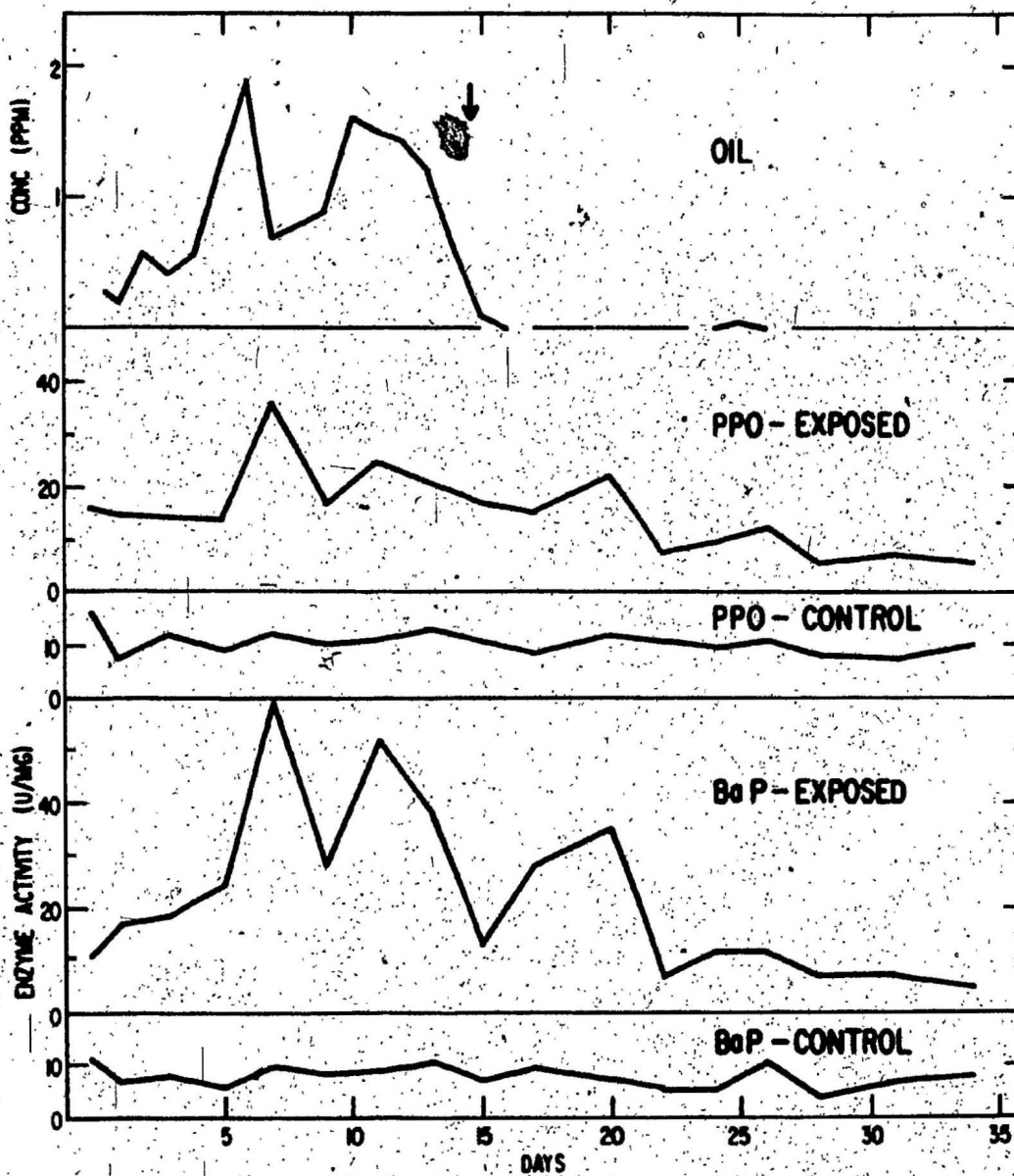


Fig. 11. Oil concentrations in the water and the corresponding eunner
hepatic aryl hydrocarbon hydroxylase activities, using the substrates
2,5-diphenyloxazole (PPO) and benzo[a]pyrene (BaP), through time.
The oil was removed at day 14, as indicated by the arrow.



was therefore examined following the force-feeding of oil or oil-contaminated Mytilus tissue.

These exposures to oil utilized a flow-through tank (Fig. 12) with three tiers of 16 compartments (each measuring 24cm x 10cm x 12cm). Flow-rate was regulated by valves on the waterline, filling the forward reservoir. Water entered the compartments through holes bored in the forward baffle and spilled into the rear drainage trough over the rear baffle.

The injection apparatus used to force-feed the oil consisted of a section of disposable pipette (8-10 cm long) connected to a 1 ml syringe barrel by rubber tubing. The pipette section used was the lower half of a 1 ml disposable plastic serum pipette. Each sample was force-fed by drawing up the desired quantity into the pipette, forcing the pipette down the fish's esophagus and injecting the sample into the gut. In comparison to the oil, homogenized Mytilus tissue (see p. 34) is much more viscous, consequently the injection apparatus had to be modified by substituting the upper third of a disposable 2 ml glass pipette for the pipette section and a 3 ml syringe barrel for the 1 ml barrel. Also the Mytilus sample to be injected was manually loaded into the syringe barrel for injection.

The force-feeding experiment involving the injection of oil into the fish gut was conducted from September 29 to October 17. In addition to the eight control fish there were three other treatment groups; seven fish were fed 0.4 ml salt water every third day, nine fish were fed 0.4 ml water soluble fraction every third day, and fourteen fish were fed 0.1 ml oil every 7 days. The water soluble fraction of the crude oil was obtained by floating 100 ml of oil on 300 ml of sea water which was

Fig. 12. The compartmentalized flow-through tank. Water entered the compartments from the trough at the back of each tier and flowed out over a baffle at the front of each tier.



continuously and slowly stirred for 24 hours with a magnetic stirrer in an aspirator flask. Samples for injection and oil concentration analysis were drawn from the water layer following a 5-10 hour non-stirring period which allowed suspended oil droplets to separate. The resultant enzyme activities (Table 9) show significant induction by oil only. That salt water does not cause induction is evidence that physical handling of the fish does not cause induction. Regardless of the material force-fed there was no significant difference in the level of activity with respect to sex of the fish.

Oiled Mytilus tissue was obtained by maintaining mussels in a 20 L flow-through tank for 7-14 days with a daily surface application of 5-7 ml of oil. These mussels were allowed to depurate for 5-10 hours in clean flowing sea water prior to shucking and any visible oil droplets were wiped from the tissue. Oiled and non-oiled Mytilus tissue was homogenized with a Sorvall Omni-Mixer, the product divided into 20 g samples, and stored at -20 C. 12 Control and 11 Experimental fish were fed 1.0-1.5 g of non-oiled or oiled Mytilus tissue respectively every 48 hours between November 1 to November 11. Table 10 indicates the amount of oil contained in the Mytilus tissue of each lot. The resultant enzyme activities (Table 11) using both substrates, BP and PPO, demonstrated significant (at least $P < 0.05$) level of induction. No sex difference in enzyme activity could be detected in the controls or experimentals.

IV Other Results Pertinent to AHH Assay Use

Data generated by the Portugal Cove August 19 field collection and surface slick oil exposure can be used to arrive at other recommendations

Table 9. Change in cunner hepatic aryl hydrocarbon hydroxylase activity, using the substrate PPO, following intermittent force-feeding of oil over a three week period.

	Mean Enzyme Activity (Std. Dev.)	Average Liver Weight g	Average Body Weight g	Liver Somatic Index % (Std. Dev.)	Sample Size
<u>Control:</u>					
Male	5.8 (2.53)	3.7	169	2.63 (0.33)	6
Female	4.6 (1.70)	3.3	174	1.81 (0.36)	2
Combined	5.5 (2.30)	3.6	170	2.16 (0.54)	8
<u>Salt Water</u>					
<u>Injection:</u>					
Male	5.1 (1.55)	3.3	130	1.62 (0.57)	3
Female	5.3 (1.51)	2.8	150	2.51 (1.12)	4
Combined	5.2 (1.40)	3.0	170	1.95 (0.87)	7
<u>Water Soluble</u>					
<u>Fraction</u>					
<u>Injection:</u>					
Male	6.1 (1.68)	2.7	144	1.87 (0.30)	5
Female	9.2 (4.76)	3.1	138	2.33 (0.34)	4
Combined	7.0 (3.12)	2.9	141	2.07 (0.38)	9
<u>Oil Injection:</u>					
Male	19.6 (9.61)	2.7	118	2.19 (0.63)	10
Female	12.8 (6.53)	2.9	140	2.05 (0.11)	4
Combined	17.7 (9.16)	2.7	124	2.15 (0.53)	14

Table 10. Quantity of oil contained in the Mytilus tissue force-fed to the cunners.

ug Venezuelan Crude Oil Equivalents/g Wet Weight *				
Sample	Trial 1	Trial 2	Average	Quantity of Oil Calculated by Difference
<u>Mytilus</u> I	1023	743	883	-
Oiled <u>Mytilus</u> I	1634	1500	1567	684
<u>Mytilus</u> II	648	697	673	-
Oiled <u>Mytilus</u> II	1535	1521	1528	855

* The numbers shown are based solely on the ultraviolet absorbance at 292 nm, which would also include endogenous (non-petroleum) UV-absorbing material. The estimate of petroleum in the oiled animals is based on the difference in UV absorbance, calculated from a standard curve, assuming no contamination in the non-oiled animals.

Table 11. Change in cunner hepatic aryl hydrocarbon hydroxylase activity using both assay substrates, BP and PPO, following intermittent feeding over a ten-day period of oil contaminated Mytilus tissue.

	Mean Enzyme Activity (Std. Dev.)	Average Liver - Weight g	Average Body Weight g	Liver Somatic Index % (Std. Dev.)	Sample Size
<u>Control</u>					
<u>PPO</u> : Male	5.6 (1.72)	5.3	188	2.79 (0.52)	7
Female	4.2 (1.28)	4.6	156	2.94 (0.96)	4
Combined	5.1 (1.66)	5.0	176	2.84 (0.67)	11
<u>BP</u> : Male	4.6 (1.74)	same	same	same	same
Female	2.6 (1.42)	as	as	as	as
Combined	3.8 (1.81)	above	above	above	above
<u>Experimental</u>					
<u>PPO</u> : Male	30.2 (8.33)	4.1	162	2.57 (0.34)	7
Female	19.8 (7.37)	3.0	135	2.31 (0.80)	5
Combined	25.9 (9.29)	3.6	151	2.46 (0.56)	12
<u>BP</u> : Male	85.2 (25.30)	same	same	same	same
Female	53.1 (14.70)	as	as	as	as
Combined	71.8 (26.50)	above	above	above	above

and conclusions.

Ease of homogenizing with a Wheaton 15 ml tissue grinder indicated that 3 g is the practical maximum amount of liver to be sampled. A graph of liver versus body weight (Fig. 13) therefore indicates the optimal fish size, for assay use, is 100-250 g.

A plot of specific activity versus liver or body weight does not show good correlation (Fig. 14 a,b). This "shot-gun" scatter may be the result of not homogenizing a standardized quantity of liver. Fish of 600 g frequently had livers weighing more than 12 g and these had to be subsampled. By using fish of 100-250 g such problems could be avoided.

Assuming a constant protein content per unit of liver, total hepatic AHH activity can be estimated by multiplying enzyme specific activity by liver weight. Plotting this estimate against body or liver weight yields an increasing linear function (Fig. 15 a,b). Total hepatic enzyme activity therefore increases linearly with both body and liver weight.

How well PPO substitutes for BP in the enzyme assay can be seen by plotting BP versus PPO enzyme specific activities for individual fish. If PPO and BP are metabolized equally well by the same enzyme or P450 system a linear relationship would be expected. The curve (Fig. 16) appears to be biphasic; it is linear up to a specific activity of approximately 25 beyond which the PPO activity seems to increase at a lesser rate.

Fig. 13. Relationship between liver weight and body weight for cunners collected from Portugal Cove on August 19. Slope = 0.016; intercept = 0.94; correlation coefficient = 0.91. (Other collections demonstrated a similar relationship).

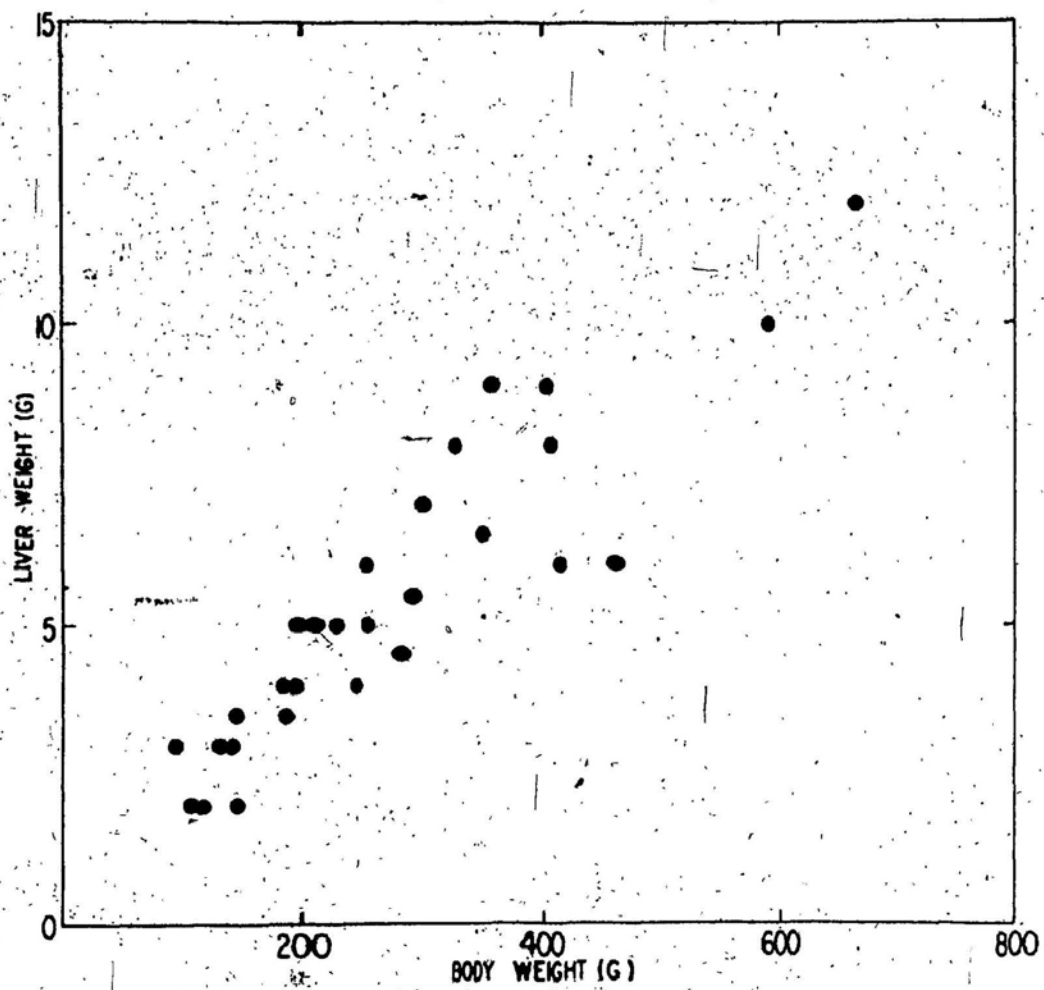


Fig. 14 (a). Relationship between specific activity (fluorescence per milligram protein) and liver weight for cunners collected from Portugal Cove on August 19. Slope = -0.67 ; intercept = 28.7 ; correlation coefficient = -0.27 . (Other collections demonstrated a similar relationship).

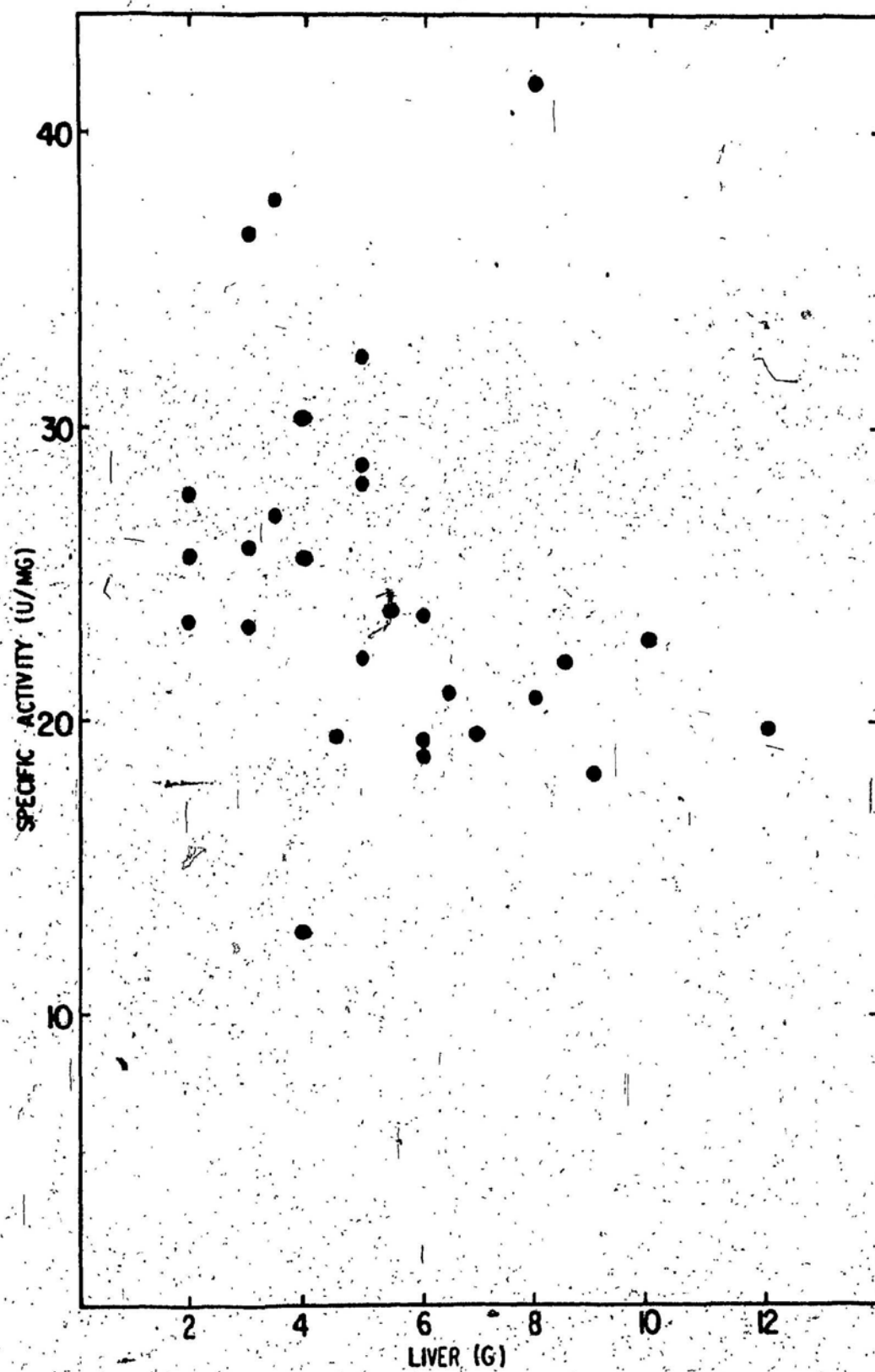
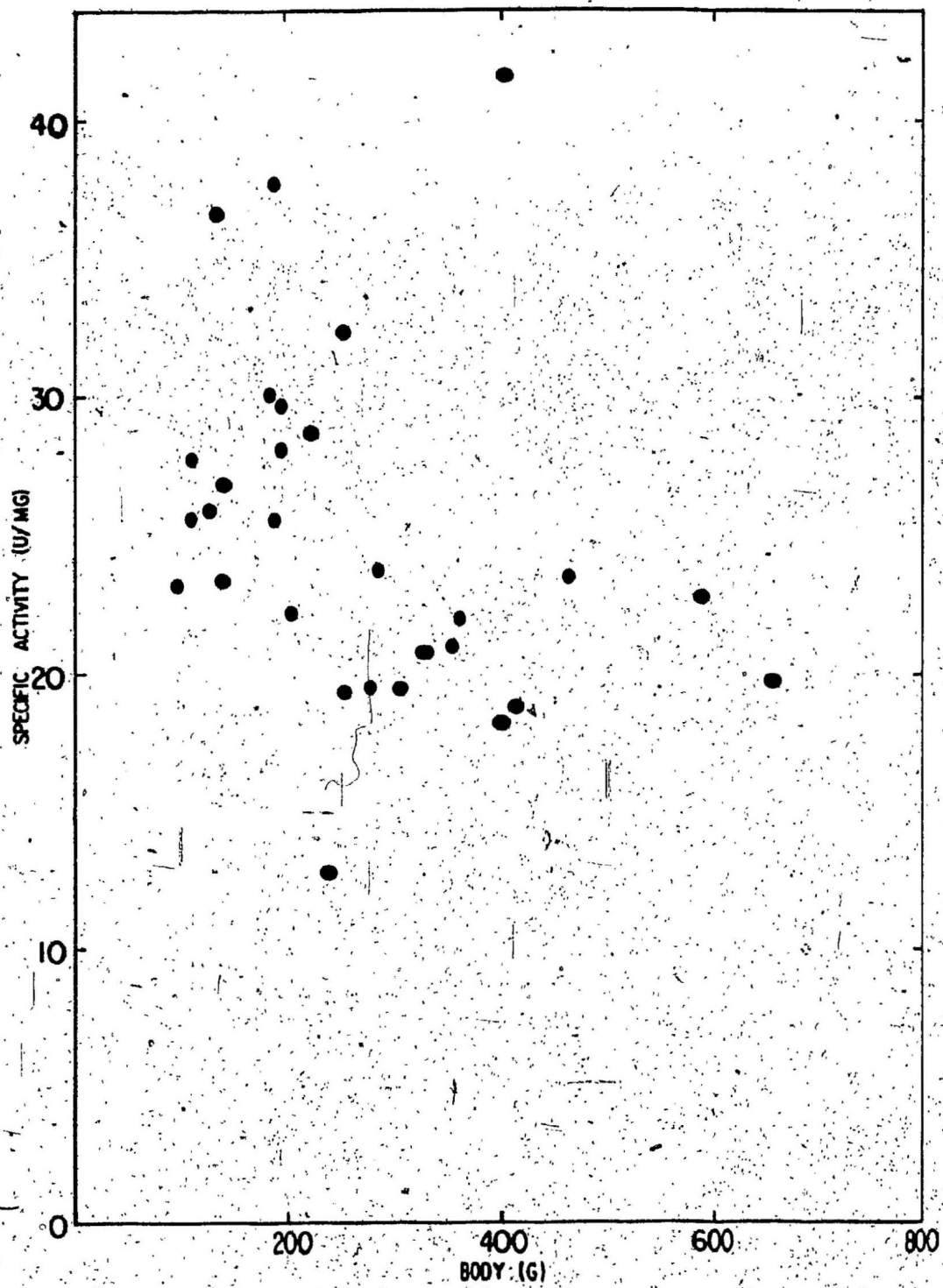


Fig. 14 (b). Relationship between specific activity (fluorescence per milligram protein) and body weight for cunners collected from Portugal Cove on August 19. Slope = -0.013 ; intercept = 28.5 ; correlation coefficient = -0.29 . (Other collections demonstrated a similar relationship).



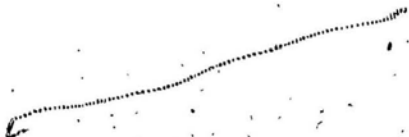


Fig. 15 (a). Relationship between estimated total aryl hydrocarbon hydroxylase activity (liver weight x specific activity) and liver weight for cunners collected from Portugal Cove on August 19.

Slope = 20.1; intercept = 21.8; correlation coefficient = 0.81.

(Other collections demonstrated a similar relationship).

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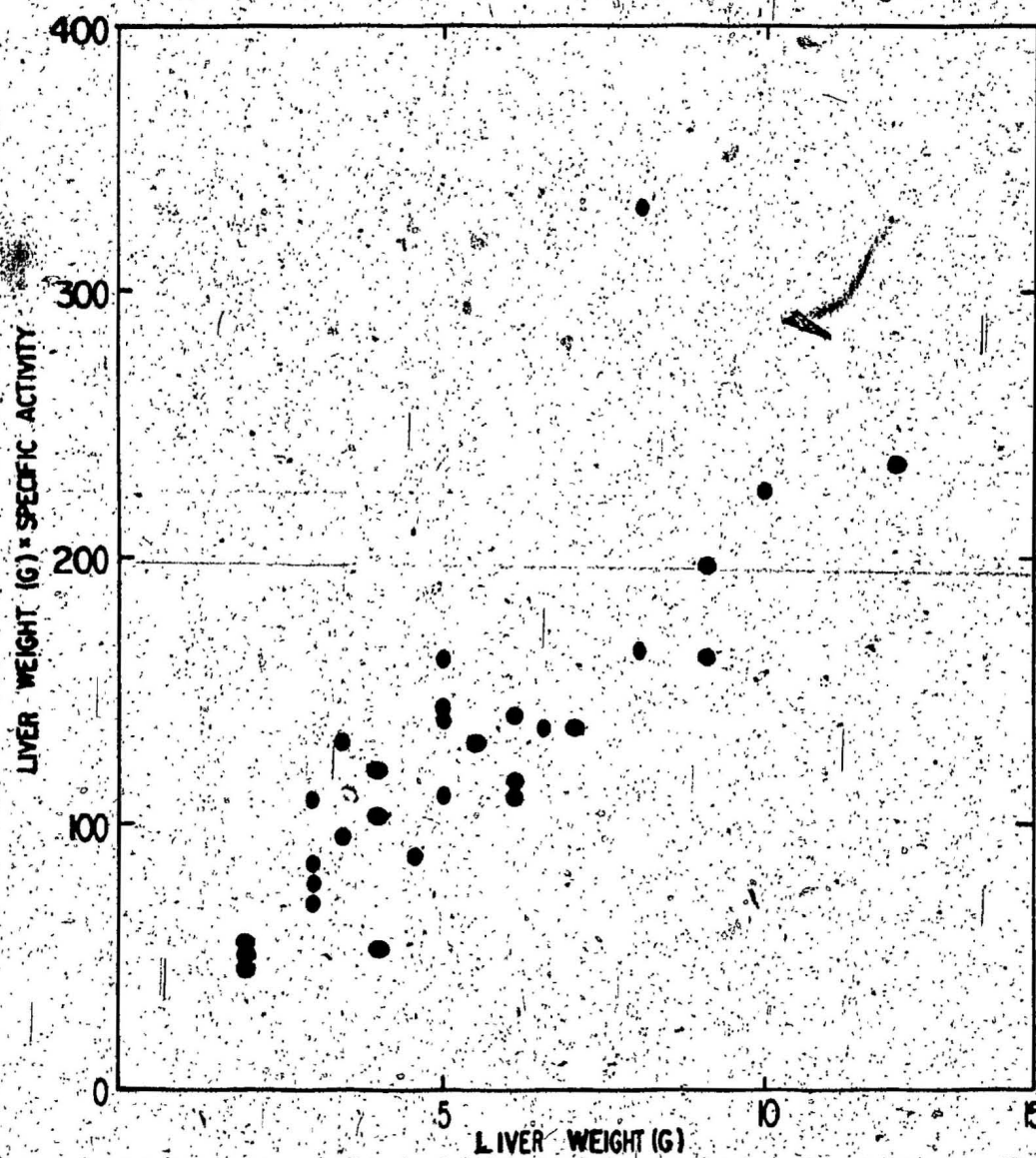


Fig. 15 (b). Relationship between estimated total aryl hydrocarbon hydroxylase activity (liver weight x specific activity) and body weight for cunners collected from Portugal Cove on August 19.
Slope = 322.9; intercept = 42.1; correlation coefficient = 0.73.
(Other collections demonstrated a similar relationship).

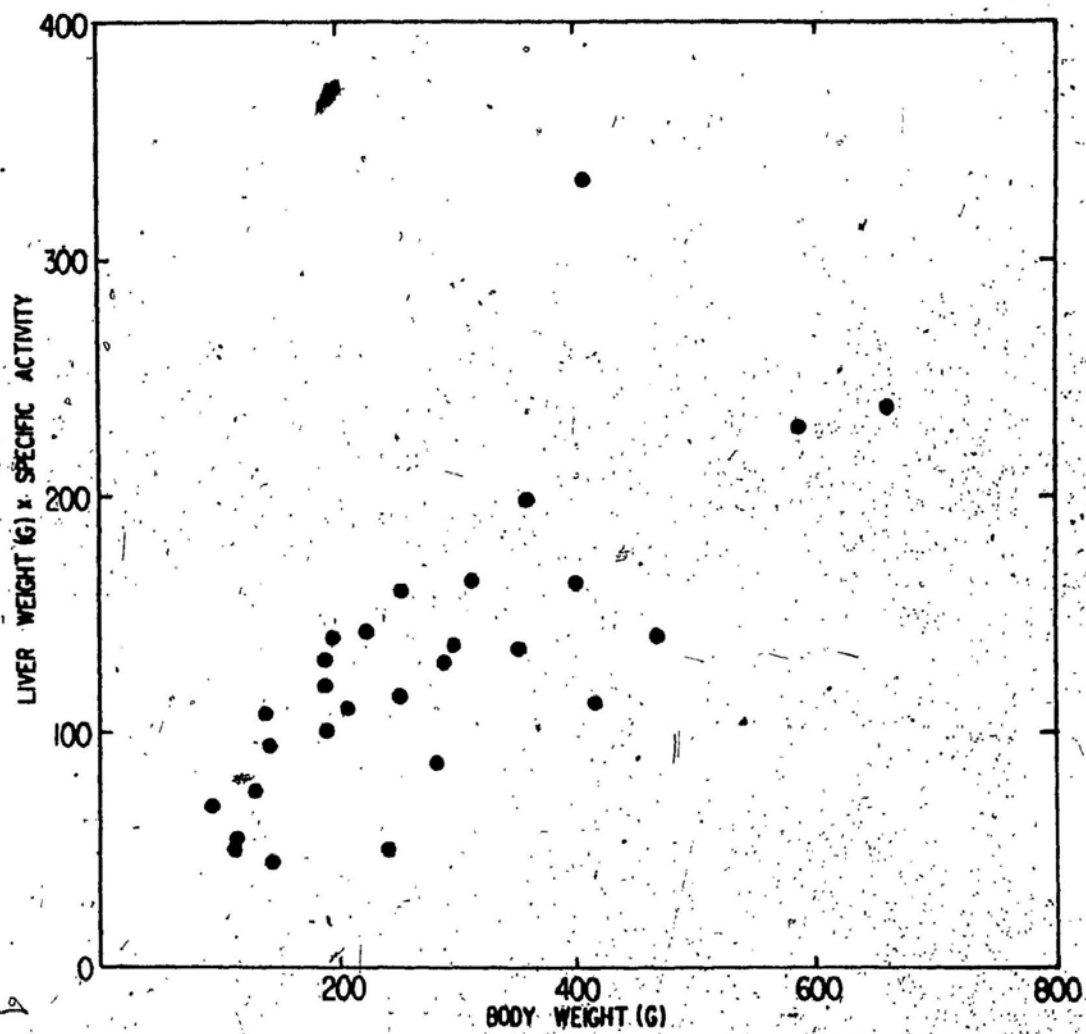
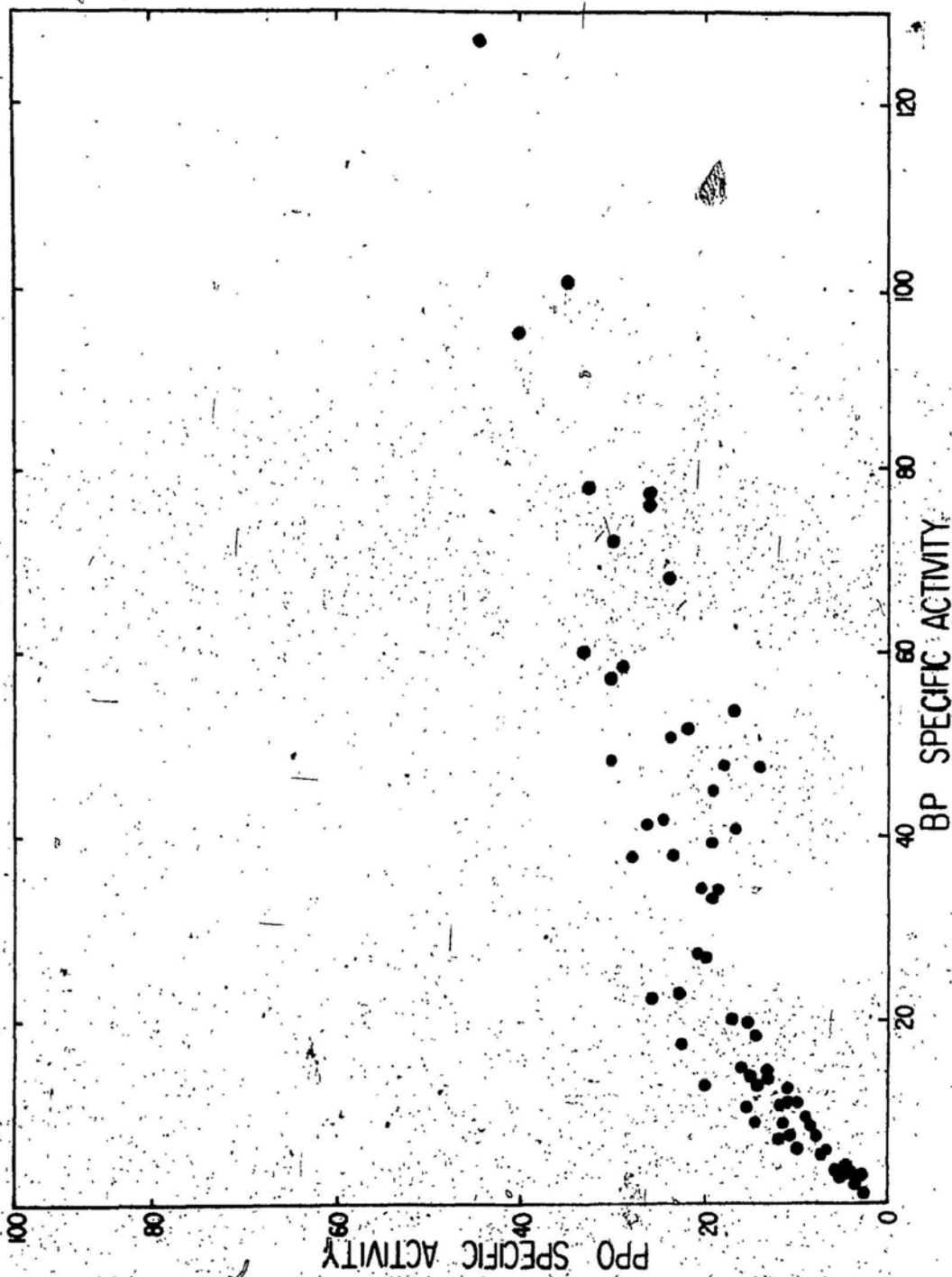


Fig. 16. Relationship between PPO and BP specific activities for individual cunners.



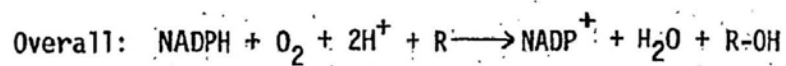
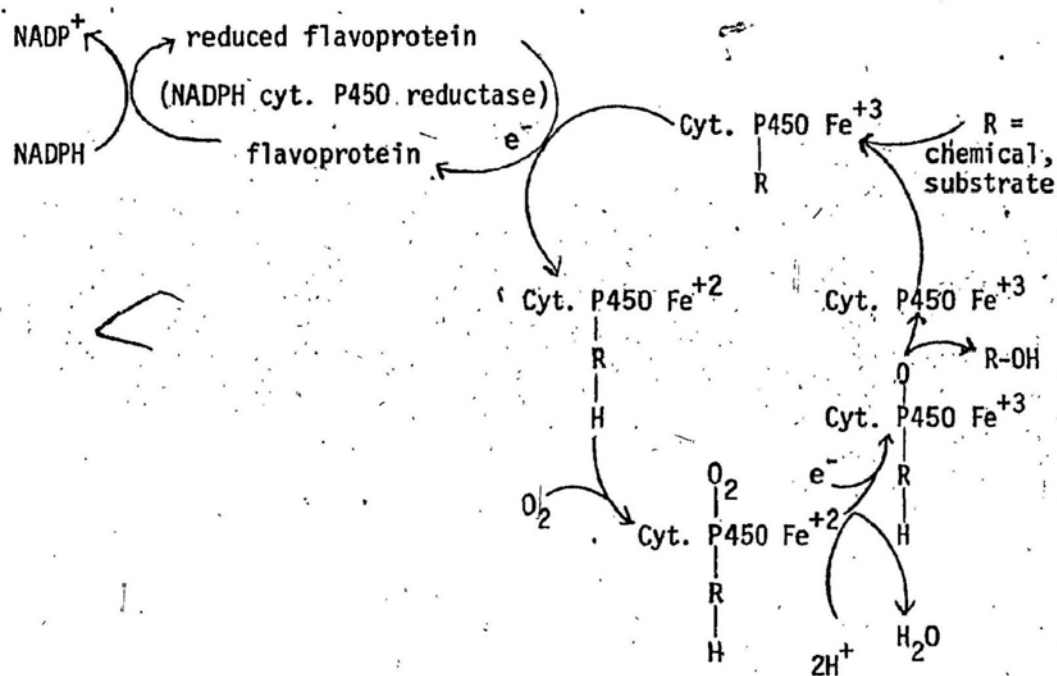
Discussion

The mixed function oxidase system presents a complex, controversial research area. In rat and rabbit liver it is thought to consist of cytochrome P450, NADPH-cytochrome P450 reductase, and lipid, likely phosphatidylcholine (Fig. 17) (Ryan *et al.*, 1975). Hydroxylation involves the binding of the aliphatic or aromatic hydrocarbon and oxygen to cytochrome P450 yielding an "active oxygen" form of P450 (Goldstein *et al.*, 1974). This complex then decomposes to produce an oxidized molecule, oxidized P450, and an equivalent of water. The complete mechanism and its components when polyaromatic hydrocarbons are the substrates comprise what is known as aryl hydrocarbon hydroxylase (AHH). Many factors such as age, sex, species, trace metal metabolism, and chemical pretreatment have been shown to affect the activity of mammalian microsomal enzymes (Conney, 1967; Maines and Kappas, 1977). If the AHH assay in cunners is to be routinely used for monitoring aquatic petroleum hydrocarbons the user must be aware of the influence these factors can have.

Sex differences in hepatic drug-metabolizing ability are known to occur in some strains of mice and gerbils, where female activity is greater than in the male, and in the rat where male activity is greater (Vesell, 1968; Maines and Westrall, 1971; Chhabra and Fouts, 1974). In rats the higher drug-oxidizing activity in the liver microsomal fraction appears to be due to an androgenic effect as castration depresses and testosterone administration enhances drug metabolism (Murphy and Dubois, 1958; Quinn *et al.*, 1958; Schenkman *et al.*, 1967). Administration of the estrogen, estradiol, has been shown to decrease drug-metabolizing activity (Murphy and Dubois, 1958; Quinn *et al.*, 1958; Inscoe and

Fig. 17. The benzo[a]pyrene (aryl hydrocarbon) hydroxylase mechanism.

(from Goldstein *et al.*, 1974).



Axelrod, 1960). AHH sex difference in extrahepatic tissues is known for rat kidney where the female has higher activity (Gurtob and Parker, 1977).

Although earlier workers (Buhler and Rasmusson, 1968; Dewaide and Henderson, 1970; Dewaide, 1971) could not demonstrate an enzymic sex difference in fish, Stegeman (1977, 1978) has shown a difference to be present when Fundulus heteroclitus and Salmo gairdneri are spawning. Such a difference was also found in the cunner with both assay substrates when examining both lab and field populations. Stegeman also found the difference to be transient, occurring only when fish are in spawning condition. Hormone cycles leading to gonadal development in rainbow trout are unknown, however, in female brown trout (Salmo trutta) development of gonads is known to be accompanied by increases in estradiol and gonadotropin levels and an increase in the production of a serum lipoprotein, vitellogenin, by the liver (Crim and Idler, 1978). However, the brown trout spawns only once per annum while the cunner like the medaka, Oryzias latipes (Briggs and Egami, 1959; Egami and Hosokawa, 1973) spawns repeatedly over several days. Reproductive cycle endocrinology is poorly known and what is known deals mainly with the one-time spawners (Crim, 1978 personal communication). Whether this cycle and the hormones involved are the same in the cunner is not known. Assuming a similar system in the cunner there are many possibilities to account for the sex difference in fish. The increasing estradiol concentration alone could depress hepatic AHH activity, or, as the hepatic hydroxylation pathway degrades estradiol (McKerns, 1969) perhaps the fish decreases hepatic hydroxylative activity to conserve energy. Peter (1978, personal communication) in reviewing hormonal influences on annual teleost gonadal cycles notes that female teleosts have substantial plasma levels of testosterone which

in some species may equal that of the males, males generally have lower estrogen levels than females but in some species the levels are similar, and an estrogen seasonal cycle correlating with testicular development is also species dependent. The production of vitellogenin is under estrogen control (Campbell and Idler, 1976; Crim and Idler, 1978). As hydroxylase activity is correlated with the hepatic microsomal concentration of protein and phospholipids (Cooper and Feuer, 1972) and may be depressed by unsaturated fatty acids (Lang, 1976) possibly vitellogenin production depresses female AHH activity. As starvation was shown to depress enzyme activity perhaps female cunners cease feeding during the spawning period. Green and Pottle (1978, personal communication), however, have observed cunners spawning within a few hours of feeding. The reason for the sex difference is therefore unclear and whether a spawning female cunner can induce as well as a male is also unknown. The experiment determining the amount of oil necessary to cause induction serves to point out that a researcher must be aware of seasonal cycles in enzyme activity in the fish species being studied.

Nutrition has also been found to influence a pollutant's toxicity (reviewed by Campbell and Hayes, 1974; Shakman, 1974). Rat liver benzo[a]pyrene hydroxylase activity has been found to decrease when the animals were starved (Wiebel and Gerboin, 1975). The effect of each nutrient class (i.e. protein, lipid, carbohydrate) is unclear although diets restricting the intake of different types of protein, individual amino acids, or lipid have resulted in lowered levels of hepatic microsomal cytochrome P450 in rats (Marshall and McLean, 1971; Fielding and Hughes, 1976; Truex *et al.*, 1977). Tissue utilization in starving fish is similar to that in mammals whereby liver glycogen stores are first

used followed by protein and fat stores (Kamra, 1966; Wilkins, 1967; Love, 1974). In cod starvation decreases the concentrations of liver phospholipid (Love, 1974) while increasing the levels of furan-containing fatty acids (Gunstone and Wijesundera, 1976). As drug-metabolizing ability is dependent on phospholipid (Copper and Feuer, 1972) and the presence of unsaturated fatty acids, *in vivo*, depresses AHH activity (Lang, 1976) either of these factors could account for decreased AHH activity upon starvation. As starvation was shown to depress cunner hepatic AHH activity and a starvation effect was demonstrated by the change in liver somatic index the nutritive status must be taken into account for routine assaying.

As a molecule decreases in molecular weight, becomes less ionized, or its partition coefficient increases, its pharmacologic effects tend to increase (Cohn, 1972). As petroleum hydrocarbons are very lipophilic, they could be taken up by any membrane (Cohn, 1972).

Benzo[a]pyrene hydroxylase activity in the rat has been found to vary depending on the tissue examined (Conney, 1967; Chhabra and Fouts, 1974; Gurtoo and Parker, 1977). It is located in highest specific activity in the liver, with lesser amounts found in the kidney, small intestine, spleen, and upon induction, can be detected in the thyroid, lung, and testis (Conney, 1967; Gurtoo and Parker, 1977). A similar distributional pattern exists in the cunner where Payne (1977, personal communication) found liver activity to be greater than kidney, and only when induced by crude oil could activity be detected in tissue from the gill, intestine, pancreas, spleen and heart.

A hypothetical defensive pattern thereby emerges. Hydrocarbons, whether they be ingested or taken up across the gills or integument, may

be biotransformed by enzymes at the site of absorption. Uptake of a chemical from the digestive tract is often limited by the bulk of the material present, metabolism by gut flora, and the effect of digestive processes on its molecular structure (Schanker, 1972; Williams, 1972). If the molecules are taken up by the respiratory, as opposed to the intestinal, route they will more likely be dispersed into body tissues due to the much longer circulatory pathway until the blood reaches the liver, the major biotransformation site. If biotransformed in the liver the metabolites will likely be secreted into the biliary tree, stored in the gall bladder, and eventually excreted into the intestine for fecal elimination or resorption (Plaa, 1972). Generally a molecular weight greater than 300 and the presence of polar groups facilitate biliary excretion but it has also been found that the extent to which this excretory route is used is species specific (Plaa, 1972). The other main excretory route is via the kidneys. By this route polar molecules having a molecular weight less than 60-70,000 are readily isolated from the blood and excreted into the urine (Cafruny, 1972). Also as enzyme activity increases the amount of active carcinogen produced increases resulting in a higher incidence of tumours (Kouri, 1976). Therefore the route of uptake may play a large role in the rate of uptake and excretion.

For both practical and physical reasons it is not possible to completely isolate and expose these routes of uptake to see if there is a measurable effect on hepatic AHH. The most common oil exposure mode is by surface slick which would expose mainly gill, though also gut, membranes to hydrocarbons. Agitation of a slick can generate balls of oil which may be ingested on the pretense of being food. Being a salt

water fish the cunner drinks sea water to facilitate osmoregulation, hence may ingest some of the oil's water soluble fraction. Direct exposure of gut membranes to hydrocarbons could therefore increase hepatic AHH activity. There may also be a food chain effect whereby ingestion of oil contaminated food could release hydrocarbons into the gut increasing hepatic AHH. Through the experiments carried out exposing cunners to oil via surface slick, oil introduced directly into the gut, and oil contaminated food, AHH activity was shown to increase. Examination of male versus female activities could not demonstrate a sex difference in hydrocarbon metabolism. The induction/de-induction experiment demonstrated that cunner AHH can be induced within twenty-four hours, the induced state remains as long as oil is present, and that de-induction is quite rapid, occurring within a week. In the field Kurelec *et al.* (1977) showed hepatic induction took about three days in Blennius pavo when the water was saturated with Diesel 2 oil and persisted at an induced activity level as long as oil was present.

The Mytilus force-feeding experiment was particularly interesting as this bivalve is a natural food of the cunner (Chao, 1973; Shumway and Stickney, 1975). As feeding oil contaminated Mytilus, which being bivalves do not possess AHH, to cunners induces their hepatic AHH, more carcinogens could be activated increasing tumour incidence. It is also interesting to note that the cunner has an abnormally high incidence of oral tumours and their incidence has been proposed as an indicator of polluted waters (Harshbarger *et al.*, 1976).

Due to the number of factors which can affect microsomal enzyme activity, especially that of the female, Mazel (1972) recommends using animals of the same age and weight and to standardize, as much as possible,

the conditions under which they are kept in order to make results as comparable as possible. Although an effect due to weight (hence age) was not shown here, taking such parameters into consideration may be worthwhile. Although for laboratory work a size range of fish could be chosen, such selection in the field may not be possible. However, if the apparatus and techniques utilized here were used routinely standardizing the amount of liver homogenized from a selected size range of fish would be desirable both with regard to procedural workup and the assay's enzyme kinetics. Though PPO is non-carcinogenic it is an adequate substitute for BP only up to a specific activity of approximately 25 beyond which BP-measured enhanced activity is not paralleled by PPO. This suggests the presence of two enzymes or multiple P450's, as found in the rabbit and rat (Guengerich, 1977), which may vary with regard to substrate specificity.

Despite aryl hydrocarbon hydroxylase having been demonstrated in the field to be a useful monitor for aquatic petroleum hydrocarbons (Payne, 1976; Kurelec, 1977; Stegeman, 1978) it is known from studies on rat/AHH that many factors can influence its activity. With regard to the cunner two such factors are the individual's sex and nutritional health. As in this species males are territorial they may not be readily captured hence a collection's sex ratio may be biased toward females whose activity is markedly depressed during the spawning season. Recording liver somatic index is important, as discrepancies in activity between collection sites may then be indicated on a nutritional basis. As cunners move to overwintering sites once the water temperature approaches 5 C and remain torpid at these sites through the winter (Green and Farwell, 1971), their usefulness as an indicator species, in Newfoundland, may be limited during

the winter due to the difficulty of capture and lack of knowledge of induction under colder conditions. For the rest of the year the cunner is abundant, readily captured in sizeable numbers, and its hardiness makes it a convenient research species. Also being a shore fish which homes and whose males are territorial add to this species' attributes as an indicator of local pollution. Another restriction on AHH utility may be the influence on activity by other pollutants. Although hepatic AHH activity in trout has been shown to not be influenced by fenitrothion, carbaryl, DDT, and Aroclor 1016 (Payne, 1976) such screening has not been done in the cunner. When the sex and nutrition factors are accounted for, induction of AHH occurred equally well in both sexes when oil was presented to the fish via surface slick, injection directly into the gut, and in the food. Providing such factors as sex, nutrition, and other pollutants are proven to have a small influence on activity in comparison to the petroleum hydrocarbon induction, aryl hydrocarbon hydroxylase is a reasonable indicator for monitoring the presence of petroleum hydrocarbons.

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